The role of different albumin preparations on production of human plasma lipoprotein-like particles in vitro

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Abstract Because we found apoprotein contamination of some high-grade commercial albumins, we studied this effect on formation of lipoprotein-like particles during lipolysis of human very low density lipoprotein (VLDL) in vitro. After a 1-hr incubation with purified bovine milk lipoprotein lipase, over 98% VLDL triglyceride was hydrolyzed in the presence of either albumin B (apoprotein-rich) or albumin C (apoprotein-poor), with a weight ratio of albumin to triglyceride of 60 to 1. Lipoproteins of density < 1.019 g/ml ("IDL"), 1.019 to 1.063 g/ml ("LDL"), and 1.063 to 1.21 g/ml ("HDL") were then isolated by ultracentrifugation. Recovery of non-triglyceride VLDL constituents in "IDL" and "LDL" was similar for albumin B or albumin C. "LDL" was the major catabolic product of in vitro VLDL lipolysis independent of the albumin used. The yield of "HDL," however, was 5- to 6-fold greater with albumin B. All lipoproteins produced with albumin B were richer in phospholipid, apoproteins C and A-I, relative to lipoproteins produced in the presence of albumin C. With albumin B, cholesterol/phospholipid molar ratios were <1 in all in vitro produced lipoproteins, but were >1 with albumin C. All these differences can be ascribed to the presence in albumin B of 0.2 mg apoprotein A-I/g albumin and 1.8 mg phospholipid/g albumin; these components were not detected in albumin C. Thus, two thirds of "HDL" recovered with VLDL lipolysis in the presence of albumin B can be accounted for by albumin itself and only one third from constituents of VLDL. Adding equivalent amounts of both apoproteins removed from albumin B and phospholipid to albumin C markedly decreased the disparities in results but addition of each alone did not. These results prove "inert" albumins serve other than as fatty acid and lysolecithin acceptors in in vitro model systems, and do influence formation of lipoproteins during in vitro VLDL catabolism.—**Deckelbaum, R. J., T. Olivecrona, and M. Fainaru.** The role of different albumin preparations on production of human plasma lipoprotein-like particles in vitro. *J. Lipid Res.* 1980. **21:** 425–434.

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Following hydrolysis of triglyceride in plasma very low density lipoproteins (VLDL), individual constituents of VLDL redistribute into other lipoprotein classes and lipoprotein-free plasma fractions. In vivo studies in animals and man (1–4) have shown that with VLDL lipolysis apoprotein B distributes to intermediate density and low density lipoproteins (IDL and LDL). As the VLDL core shrinks and excess surface components are removed, C apoproteins distribute largely to high density lipoproteins (HDL) (2, 3, 5). In vitro systems have also been introduced for the study of VLDL catabolism and subsequent fate of non-triglyceride components. In vitro systems have largely used post-heparin plasma (1, 6–8) or purified lipoprotein lipases (9–12) to obtain triglyceride lipolysis, or employed organ perfusions as a source of lipoprotein lipase (13–16).

In many in vitro studies commercial albumin preparations are used either for maintaining oncotic pressure, or as acceptors for fatty acid and lysolecithin released upon degradation of triglyceride-rich lipoproteins (1, 6–12, 14–17). In some papers the specific source of albumin is never identified. Albumin preparations are generally accepted as being pure, and aside from their role in binding fatty acid and lysolecithin, supposedly are inert in the experimental model under examination. We have recently reported, however, that some high-grade commercial "fatty acid-poor" and "fatty acid-free" albumins contain apoproteins in appreciable amounts (18).

To determine whether different albumins influence

Abbreviations: VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins. (In vitro-produced particles floating in the IDL, LDL, and HDL density ranges are enclosed in quotation marks, as "IDL," "LDL," and "HDL," respectively. Native lipoproteins isolated from plasma are identified without quotation marks.) SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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results of experiments on VLDL metabolism we undertook studies comparing the effects of two commercial albumins widely used in in vitro models of lipoprotein metabolism. We used a model in which human lipoprotein-like particles are produced in vitro from VLDL via the action of purified bovine milk lipoprotein lipase (9). In this system fatty acid-poor albumin is used solely as an acceptor for fatty acid and lysolecithin released upon VLDL lipolysis.

We report that, depending upon the commercial albumin used in the incubation system, appreciable differences are observed in redistribution of individual constituents of VLDL after triglyceride lipolysis. Differences are mainly quantitative and relate to the quantity of components partitioning to LDL and HDL density ranges following lipolysis, but some qualitative differences exist as well. Our results suggest that it is not apoprotein contamination alone that is responsible for these differences, but rather the synergistic activities of these apoproteins with phospholipids also present in the commercial albumin.

METHODS

Albumin preparations

To compare possible differences between commercial albumins, two bovine albumins were chosen. The albumin preparations were obtained from Sigma Chemical Co. (St. Louis, MO) and Pentex, Miles Laboratories (Kankakee, IL). They were the highest grade of "fatty acid-free" or "fatty acid-poor" available from each company, and only albumins with the same respective lot number were used for these studies. Since there may be differences in results obtained with various batches, we prefer arbitrarily to designate the two albumins as B and C (albumin B, lot no. 28; albumin C, lot no. 7442). Albumin B has been previously shown to contain almost 200 μg of bovine apoprotein A-I (apo A-I) per 1 g of albumin, whereas albumin C contained undetectable amounts of apo A-I as judged by radial immunodiffusion (18).

Isolation of native plasma lipoproteins

Blood was collected in vacuum packs containing 1 mg/ml disodium EDTA from fasting (14–16 hr) normolipemic or type IV hypertriglyceridemic males. Plasma was immediately separated and individual lipoprotein classes were prepared by repetitive salt density ultracentrifugation using density solutions of NaCl and KBr as previously described (19). VLDL was isolated at plasma density d 1.006 g/ml, IDL between 1.006–1.019 g/ml, LDL between 1.019–1.063 g/ml, and HDL between salt densities of 1.063–1.210 g/ml. Isolated lipoproteins were washed once in NaCl-KBr solutions of appropriate density. All solutions contained 1 mM EDTA and were adjusted to pH 8.5 using NH_4OH. All centrifuge runs were at 4°C for 19–21 hr (HDL was isolated over 43–45 hr) in Beckman 40.3 or 50 Ti rotors at 40,000 rpm and 50,000 rpm, respectively. Any chylomicrons remaining in VLDL were removed by a single spin of the VLDL in a Beckman SW-41 rotor at 40,000 rpm for 30 min at 10°C. After isolation, all lipoproteins were dialyzed in the dark for 24 hr against normal saline (0.15 M NaCl, 1 mM EDTA, pH 8.5, 1:100 v:v) at 4°C with a minimum of three changes of dialysate. For storage, lipoproteins were overlayered with nitrogen and stored in the dark at 4°C.

Preparation of in vitro-produced lipoproteins

VLDL was obtained as described above. Purified bovine milk lipoprotein lipase was isolated by heparin-Sepharose affinity chromatography by methods detailed elsewhere (20). The enzyme was stored frozen in 5 mM veronal-HCl, 5 mM sodium deoxycholate, pH 7.4, at a concentration of 300 μg per ml. The activity of the purified enzyme in the incubation buffers described below was 300–500 units of lipolytic activity per mg of protein (1 unit = 1 μmol of fatty acid released per minute at 24°C). For incubation, sufficient enzyme was placed in each tube to insure that maximal release of fatty acid released by 60 min incubation (usually 10–40 μl depending on amount of substrate VLDL and volume).[4]

Incubation of VLDL with lipase was performed in parallel under identical conditions for both albumins B and C using single VLDL preparations. Conditions of incubation were as previously described (9), with each ml of incubation solution containing 6% albumin in 0.2 M Tris-HCl buffer (Sigma Chemical Co., St. Louis, MO), pH 8.4 in 0.08 M NaCl, 2 units of heparin (Evans Medical Ltd., Liverpool, England) and 1 mg VLDL triglycerides. Following 1 hr incubation at 37°C, enzyme activity was inhibited by addition of NaCl solution which raised the salt density of the incubation mixture to 1.019 g/ml (or in some experiments to 1.063 g/ml with NaCl-KBr). Particles produced after VLDL lipolysis were then separated by

[4] Sodium deoxycholate is added to the enzyme solution as it is advantageous in maintaining enzyme activity during shipping and storage. Parallel VLDL incubations with lipoprotein lipase in incubation mixtures containing no deoxycholate and mixtures with up to twofold the bile salt concentration ever reached in the present study were performed. No significant effects of this bile salt on lipid or protein distribution or composition of in vitro-produced lipoproteins were noted using the identical model system outlined in this paper (Shlomo Eisenberg, personal communication).

ultracentrifugation by methods identical to those described above. In vitro-produced lipoproteins were isolated at density ranges corresponding to their native counterparts, i.e., "IDL" at d < 1.019 g/ml, "LDL" at d 1.019–1.063 g/ml and "HDL" at d 1.063–1.210 g/ml.

Lipoproteins isolated in vitro were dialyzed and stored as described above and further studies were performed within 10 days of isolation.

In some experiments to remove lipid found to be associated with the commercial albumins, albumin was delipidated in ethanol–ether 3:1 (v/v) according to Scanu and Edelstein (21).

To remove lipid-binding apoproteins from the commercial albumins, 300 mg of albumin was incubated with 10 μM egg yolk phosphatidylcholine (Makor Co., Jerusalem, Israel) prepared as single lamellar vesicles according to the method of Suurkuusk et al. (22). The albumin and phosphatidylcholine were incubated in 6 ml of 0.15 M NaCl containing 0.05 M Tris-HCl, pH 7.5, for 2 hr at 23°C. The density was then raised to 1.210 g/ml with solid KBr and the mixture was centrifuged in a Beckman 40.3 rotor for 48 hr at 40,000 rpm at 4°C. The top fraction (d < 1.21 g/ml) containing the lipid-binding apoproteins and the bottom fraction (d > 1.21 g/ml) were washed at the same density by subsequent ultracentrifugation. The lipid-protein complexes removed in this way from albumin B were then delipidated and the proteins used for further incubation. Similarly, egg yolk lecithin was added to albumin incubation mixtures in amounts equivalent to those removed by delipidation of albumin B.

Analyses

For lipid analysis, lipids were extracted from protein-containing fractions by chloroform–methanol 2:1 (v/v) (23). Phospholipid content was determined by the method of Bartlett (24), total cholesterol by the procedure of Chiamori and Henry (25) and triglyceride by the Autoanalyzer method (26). The content of free fatty acids was measured by radiochemical assay (27) in extracts prepared by Dole's method (28). Relative contribution of individual phospholipids to total phospholipid, and free cholesterol and cholesteryl ester to total cholesterol, were determined after separation of individual lipid classes by thin-layer chromatography using methods previously described (6). Lipoprotein–protein content was determined by the method of Lowry et al. (29) using bovine serum albumin as a standard. Sodium dodecyl sulfate (0.1%) polyacrylamide gel (10%) electrophoresis (SDS-PAGE) was performed according to the procedure described previously (30) adapted from Weber and Osborn (31). The samples were denatured by heating at 90°C for 3 min in the presence of 1% SDS and 5% 2-mercaptoethanol prior to electrophoresis.

Electron microscopy

Lipoproteins were negatively stained with 2% sodium phosphotungstate, pH 7.4, on collodion carbon-coated copper grids and sized as described previously (9). Electron micrographs were obtained with a Phillips 300 electron microscope, at instrument magnification of 63,000.

RESULTS

Effect of different albumins on the fate of VLDL constituents after lipolysis

Almost all of the VLDL–triglyceride was hydrolyzed during one hour incubation of VLDL with lipoprotein lipase in the presence of either albumin B or albumin C. In five parallel experiments, 98.1 ± 0.6% and 99.2 ± 0.3% (mean ± S.E.M.) of the original VLDL–triglyceride was hydrolyzed with albumin B and albumin C, respectively.[5] With both albumins remaining triglyceride was recovered mainly in the "IDL" and "LDL" density ranges. With either albumin maximum fatty acid release was reached 35–40 min after addition of lipase.

The redistribution of the non-triglyceride VLDL constituents in in vitro lipoproteins produced following triglyceride lipolysis is outlined in **Table 1.** With each albumin the total amounts of "IDL" and "LDL" generated after VLDL lipolysis were similar. "LDL" remained the major catabolic product of in vitro VLDL lipolysis and accounted for about 30% of the initial nontriglyceride VLDL constituents independent of the albumin used. In marked contrast, when albumin B was used the total yield of "HDL" was six times greater than with albumin C.

With VLDL–triglyceride lipolysis the amount of original VLDL phospholipid associated with in vitro-produced lipoproteins (d < 1.21 g/ml) was greater with albumin B than C (64.2 ± 16.0% and 24.5 ± 2.9%, respectively). With both albumins, 90% of the remaining phospholipid was recovered in the d

[5] Because free fatty acid released during VLDL lipolysis is derived both from phospholipid and triglyceride, triglyceride hydrolysis was determined by recovery of triglyceride in the in vitro-produced lipoproteins and infranates. Determination of free fatty acid released during incubation always resulted in slightly more than could be accounted for by the initial amount of triglyceride, presumably because of concurrent phospholipid hydrolysis. Thin-layer chromatography showed triglyceride lipolysis to be complete; in only one experiment were traces of mono- and diglycerides noted.

TABLE 1. Effect of different albumins on redistribution of VLDL constituents into in vitro-produced lipoproteins after triglyceride hydrolysis

Lipoprotein	Albumin	Lipids: Cholesteryl Ester	Lipids: Free Cholesterol	Lipids: Phospholipid	Protein	Total Non-triglyceride VLDL Constituents
"IDL"	B	6.8 ± 1.7[a]	4.9 ± 3.3	3.3 ± 0.8	4.8 ± 1.3	4.0 ± 0.6
	C	4.7 ± 1.7	3.1 ± 1.0	1.9 ± 0.5	2.1 ± 0.7	2.9 ± 1.0
"LDL"	B	44.8 ± 7.4	33.3 ± 4.0	28.7 ± 4.5	28.7 ± 5.1	30.5 ± 6.0
	C	42.9 ± 2.3	46.9 ± 5.1	19.9 ± 2.1	25.2 ± 2.2	31.0 ± 1.4
"HDL"	B	12.8 ± 4.8[b]	33.6 ± 7.4[b]	32.2 ± 13.4[b]	48.0 ± 8.6[b]	19.6 ± 3.9[b]
	C	2.1 ± 1.0	5.8 ± 4.3	2.7 ± 3.2	3.3 ± 1.2	3.1 ± 1.3

[a] Percent recovery of non-triglyceride VLDL constituents following VLDL incubation with lipoprotein lipase in vitro, where the original amount of each individual VLDL constituent is taken as 100%. A typical incubation would include 45.00 mg VLDL triglyceride, 7.66 mg VLDL cholesteryl ester, 3.21 mg VLDL free cholesterol, 8.75 mg VLDL phospholipid, and 8.31 mg VLDL apoprotein. All results were obtained from five parallel incubations from separate VLDL donors. Results are expressed as mean ± S.E.

[b] The difference between the means obtained with albumin B and C are significant ($P < 0.05$) for each constituent in the "HDL" lipoproteins only (Student's t test).

> 1.21 g/ml fraction, mainly as lysolecithin (~75%),[6] and 10% in the infranates of the washes of "IDL" and "LDL". The total recovery of phospholipid was 109.6 ± 5.5% and 82.9 ± 10.8% of initial VLDL phospholipid in the incubation mixture with albumin B or C, respectively. This represents 32% additional phospholipid recovery with albumin B and indicates that albumin itself may be an exogenous source of phospholipid. Recoveries of total cholesterol, free plus esterified, showed a similar trend but with smaller differences: 89.0 ± 11.0% versus 72.1 ± 4.7% for albumins B and C, respectively. The infranates for incubations with albumins B and C, respectively, contained 16.7 ± 1.7% and 19.1 ± 1.0% of the initial VLDL total cholesterol. (With either albumin, one third of infranate cholesterol was found in the d > 1.21 g/ml infranate, one half in the infranate of the "LDL" wash, and the remainder with the infranate of the "IDL" wash.)

The relative weight composition of substrate VLDL and post-lipolysis particles isolated in each lipoprotein density class, are compared for albumins B and C in **Table 2.** Consistent differences in composition were observed between both "LDL's" and "HDL's" obtained

[6] The phospholipid composition of the d > 1.21 g/ml infranates obtained with incubations with albumin B and C, respectively, are as follows: lysolecithin 73.8 ± 6.2% and 77.1 ± 5.5%; sphingomyelin 4.1 ± 4.1% and 0%; phosphatidylcholine 22.2 ± 4.0% and 22.9 ± 5.5%. (Means ± S.E. of four experiments with albumin B, and five experiments with albumin C.)

TABLE 2. Composition analysis of substrate VLDL and in vitro lipoproteins produced in incubations with albumin B and albumin C

Lipoprotein	Albumin	Triglyceride	Cholesteryl Ester	Free Cholesterol	Phospholipid	Total Protein	Cholesterol/ Phospholipid Molar Ratios[a]	Phosphatidyl-choline/ Sphingomyelin Ratio[b]
VLDL		61.7 ± 2.2[c]	10.5 ± 1.0	4.4 ± 0.4	12.0 ± 1.6	11.4 ± 0.6	0.72	4.0 ± 0.3
"IDL"	B	22.8 ± 6.3	32.1 ± 3.5	8.1 ± 1.3	17.1 ± 1.6	19.9 ± 1.6	0.93	2.4 ± 0.3[d]
	C	18.3 ± 5.8	37.5 ± 3.9	10.8 ± 1.1	16.7 ± 1.4	16.6 ± 0.6	1.27	1.6 ± 0.3
"LDL"	B	3.9 ± 1.0	36.8 ± 1.5	10.5 ± 0.8[d]	26.0 ± 0.7[d]	22.8 ± 1.2	0.79	2.1 ± 0.2
	C	2.2 ± 1.0	38.8 ± 1.2	18.0 ± 1.9	18.5 ± 1.7	22.5 ± 1.0	1.91	1.6 ± 0.3
"HDL"	B	0	11.0 ± 1.4[d]	12.5 ± 1.8	30.0 ± 2.6[d]	46.6 ± 2.9[d]	0.82	1.7 ± 0.2[d]
	C	0	18.6 ± 1.4	16.9 ± 1.7	24.6 ± 1.7	39.8 ± 3.9	1.35	2.9 ± 0.4

[a] Ratio calculated from means of free cholesterol and phospholipid listed on left side of this table with molecular weights of free cholesterol and phospholipid as 387 and 760, respectively.

[b] Analysis of phospholipid showed that almost all phospholipid in VLDL and in vitro lipoproteins was phosphatidylcholine and sphingomyelin with very small amounts (<2%), if any, of lysolecithin.

[c] Composition is expressed as relative weight composition (% of total lipoprotein mass). All results are mean ± S.E. from five parallel incubations from separate VLDL donors.

[d] Significant difference between means obtained with albumins B and C, $P < 0.05$ (Student's t test).

when different albumins were used. Both "LDL" and "HDL" recovered after VLDL triglyceride hydrolysis in the presence of albumin B were consistently richer in phospholipid, and poorer in free and esterified cholesterol than those obtained with albumin C. "IDL's" recovered in these incubations show similar but smaller differences (Table 2). The free cholesterol to phospholipid molar ratio was greater than 1 in each lipoprotein density class whenever albumin C was used and less than 1 when albumin B was present. Thus, lipoproteins produced in incubations with albumin C were not only enriched in free cholesterol but also contained less phospholipid to solubilize this free cholesterol.

Differences in protein composition were also related to a particular albumin. As judged by SDS-PAGE, "LDL" and "HDL" obtained in the presence of albumin B consistently had more apoprotein A-I and C apoproteins than their counterparts produced with albumin C (**Fig. 1**).

Studies of electron micrographs of "LDL" produced with either albumin showed a population made up of mainly spherical particles as described in our earlier study (9). The mean diameter of "LDL" was 225 ± 20 Å (mean ± S.D.) and 225 ± 25 Å with albumin B and C, respectively. The appearance of a minor population of electron lucent sacs (liposomes) and occasional discoidal structures, previously reported to represent excess phospholipid in "LDL" (9), seemed diminished when albumin C was used but the differences could not be quantitated. With either albumin, "HDL" contained discoidal particles resembling nascent HDL (32). These particles measured between

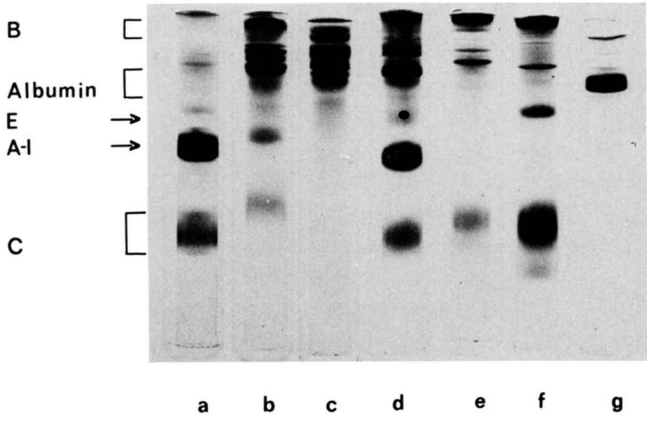

Fig. 1. SDS-PAGE of (a) human plasma HDL, (b) in vitro "LDL" produced with albumin B, (c) in vitro "LDL" produced with albumin C, (d) in vitro "HDL" produced with albumin B, (e) in vitro "HDL" produced with albumin C, (f) substrate VLDL, (g) bovine albumin B. In each case, 0.04 mg of total apoprotein was applied to the gels. Apoprotein bands were identified on the basis of their mobilities relative to purified apoproteins isolated by column chromatography.

TABLE 3. Lipids and apoprotein A-I in bovine albumins B and C

	Albumin B	Albumin C
Apoprotein A-I (μg)	197	not detected[a]
Phospholipid (μg)	1883[b]	not detected[a]
Total cholesterol (μg)	730[c]	156
Free fatty acid (μeq)	0.97	1.62

[a] Our analysis would accurately detect the following minimal amounts of each constituent: apoprotein A-I, 100; phospholipid, 21 (μg/g dry albumin).

[b] This phospholipid contained 52.7%, 36.8%, and 10.4% of phosphatidylcholine, sphingomyelin, and lysolecithin, respectively.

[c] Of this, 31.5% was free cholesterol. Thus, as calculated in Table 2, the free cholesterol/phospholipid molar ratio was 0.24 in albumin B. The percent of free cholesterol in albumin C was not determined because of the large quantities of albumin needed to perform these measurements.

Results are expressed per gram of dry albumin. Each value is the mean of three determinations.

150 and 400 Å in diameter and 60 to 90 Å in thickness, values similar to those reported in our earlier study (9). Therefore, independent of the albumin used, VLDL lipolysis produced a population of mainly spherical particles in the "LDL" density range similar to plasma LDL and, in the "HDL" ranges, discoidal particles resembling nascent HDL.

Albumin composition and its effect on the fate of VLDL constituents after triglyceride lipolysis

The two albumin preparations used in the incubation mixtures contained measurable amounts of cholesterol and free fatty acids, and albumin B contained appreciable amounts of phospholipid as well (**Table 3**). In addition, lipid-binding proteins were recovered upon incubation with phosphatidylcholine vesicles from both albumins. Polyacrylamide gel electrophoresis showed that the lipid-binding proteins of albumin B co-electrophoresed with bovine apoprotein A-I and C apoproteins (**Fig. 2**). Lipid-binding proteins, removed from albumin C showed no apoprotein A-I by SDS-PAGE but did have a broad band migrating in the region of bovine C apoproteins (Fig. 2). With both albumins the major protein removed with the lipid-binding protein fraction was albumin itself. From the values given in Table 3, we can calculate that when albumin B is used in incubations, albumin contributes an additional 50% to VLDL phospholipid and 20% to total cholesterol. Albumin B also adds apoprotein A-I in amounts greater than 10% of the original non-B apoproteins present in the VLDL used for incubation. Moreover, C-apoprotein and perhaps other unidentified apoproteins were added to the incubation when albumin B was used although we cannot definitely quantitate these at this time.

The disparities between experiments performed

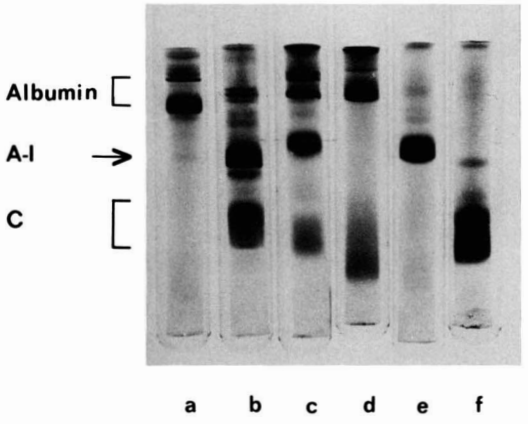

Fig. 2. SDS-PAGE of (a) bovine albumin B, (b) bovine plasma HDL, (c) lipid-binding proteins removed from albumin B, (d) lipid-binding proteins removed from albumin C, (e) bovine apoprotein A-I, (f) bovine C apoproteins. In each case, 0.10 to 0.11 mg of total protein was applied to the gel.

with the two albumins may be due to these impurities. To identify the main causes of these differences we removed phospholipids and lipid-binding proteins from albumin B, and added them to albumin C in equivalent amounts (**Table 4**). When lipid-binding apoproteins, removed from albumin B (see Methods), were added to incubations with albumin C (in equivalent concentrations to what is present in albumin B) an increase in total phospholipid recovered in the "HDL" density range occurred. Nevertheless, relative to incubation with albumin B, the amount of "HDL" phospholipid generated in the lipolytic model system was small. Similarly, although addition of phospholipid (as lecithin) to albumin C incubations had some effect on "HDL" production it was small. Simultaneous addition of both phospholipid and lipid-binding apoproteins to albumin C incubations had a much greater effect than either factor individually (Table 4).

Removing contaminating lipid and/or apoprotein from albumin B was not as helpful. Despite repeated delipidation of albumin B (see Methods), we are able to remove only 80% of the phospholipid originally present. (Using other solvents for more complete delipidation, e.g., chloroform–methanol 2:1, produced irreversible denaturation of the albumin.) Paradoxically, delipidated albumin B consistently

TABLE 4. Effects of addition or removal of albumin contaminants on the amount of "HDL" phospholipids and protein recovered after VLDL lipolysis in vitro

Albumin and Conditions of Incubation	Percent Recovery in in vitro "HDL"	
	Phospholipid	Protein
B alone	100[a]	100
B delipidated[b]	138.6 ± 5.0	195.4 ± 15.6
B delipidated − lipid binding proteins[c]	73.5 ± 9.8	95.6 ± 27.1
B delipidated + phosphatidylcholine	119.6 ± 11.6	112.5 ± 8.6
C alone	7.8 ± 1.2	13.2 ± 11.0
C + phosphatidylcholine	11.9 ± 3.5	14.0 ± 6.8
C + lipid binding proteins removed from albumin B	18.4 ± 4.1	27.5 ± 4.4
C + phosphatidylcholine + lipid binding proteins	58.8 ± 6.9	48.7 ± 5.4

[a] For each incubation the absolute amount of phospholipid and protein recovered in in vitro "HDL" when albumin B alone was used was taken as 100%. The recoveries of phospholipid and protein in simultaneous incubations under the other conditions described were then calculated as a percent recovered from incubations with albumin B alone. Results are the means ± 1 S.D. of two or three separate experiments.

[b] Delipidation of albumin B resulted in removal of only 80% of contaminating phospholipid.

[c] Removal of lipid binding proteins was incomplete as judged from the presence of apoprotein A-I in the in vitro-produced lipoproteins.

Incubation conditions were as described in the Methods section, except for addition or subtraction of lipid and/or lipid binding apoproteins to or from the mixtures in amounts approximating their content in commercial albumin used. Lipid binding proteins were removed from albumin B by addition of egg yolk phosphatidylcholine to albimin B and then centrifuging in the ultracentrifuge at d = 1.21 g/ml as described under Methods. Following delipidation of the top fraction, 5 mg of this recovered protein was added back to the incubation mixture for every gram of albumin used. This is the figure previously determined to be the total amount of protein floating up from albumins B after incubation with phospholipid liposomes (18). These proteins and/or the egg yolk phosphatidylcholine were added in Tris-NaCl buffer so that concentrations and volumes of the incubation mixture were unchanged. After 1 hr incubation the density of the mixture was immediately raised to 1.063 g/ml so that in vitro "IDL" and "LDL" were isolated together. In vitro "HDL" was then isolated as usual between densities 1.063–1.210 g/ml.

produced even greater quantities of "HDL" relative to untreated albumin B. Flotation of lipid-binding protein from albumin B followed by delipidation resulted in a lower yield of "HDL", but nevertheless recovered "HDL" was far greater than when albumin C was used alone. Similar to the situation with attempts at full lipid removal, apoprotein A-I could not be removed completely and measurable amounts were still noted (by SDS-PAGE) in in vitro lipoproteins produced with albumin B treated to remove lipid-binding proteins. Therefore, manipulation of the phospholipid and/or apoprotein content of the commercial albumins resulted in only partial elimination of disparities between these two commercial albumins in production of VLDL post-lipolysis lipoproteins.

To estimate the contribution of albumin-derived phospholipid and apoprotein to "HDL", incubations were performed with only albumin and lipoprotein lipase, but no VLDL in the incubation tube. Results were compared to parallel experiments in which VLDL was incubated with albumin B in the presence and in the absence of lipoprotein lipase. Following sequential separation at densities of 1.063 and 1.21 g/ml, protein and phospholipid recovery in the "HDL" density range was determined. With only albumin and lipase (but no VLDL) in the incubation tube, protein and phospholipid recoveries in the "HDL" density range were 66.0 and 63.7 percent, respectively, of that recovered when VLDL was hydrolyzed in the presence of albumin B. (At the same time, neither phospholipid nor protein were recovered above densities of 1.063 g/ml without VLDL in the incubation system.) Almost identical yields of "HDL" were obtained when albumin B alone or albumin B with VLDL were incubated in the absence of lipoprotein lipase. These results indicate that two thirds of the "HDL" phospholipid and protein recovered when VLDL is hydrolyzed in the presence of albumin B may derive from the albumin itself, and only one third from constituents of VLDL. Nevertheless, the amount of "HDL" produced from VLDL constituents was still greater when albumin B rather than C was used for incubation.[7]

[7] This can be determined from Table 4 if the percent recovery of "HDL" protein or phospholipid with albumin C is compared to a figure of 33% for "HDL" generated in VLDL incubations with albumin B, assuming that two thirds of the "HDL" can be derived from contaminants of the albumin. Since we measured only mass amounts, we cannot differentiate albumin from VLDL-derived components once they relocate to in vitro lipoprotein density classes. However, "HDL" formed during VLDL lipolysis with albumin B does contain *bovine* apoprotein A-I derived from the albumin as judged by double immuno-diffusion. Human apoprotein A-I does not cross-react with the antibodies to bovine apoprotein A-I (Fainaru, M. and R. Deckelbaum. Unpublished data).

DISCUSSION

The present study emphasizes that albumins used for a wide variety of lipid-related experimentation may affect results in ways other than their role as fatty acid and lysolecithin acceptors or osmotically active agents. Experiments were undertaken to determine whether recently reported apoprotein contamination of some commercial albumins may influence formation of lipoprotein particles after VLDL lipolysis in vitro. We demonstrated that redistribution of VLDL constituents and composition of lipoprotein particles produced following VLDL triglyceride lipolysis in vitro show consistent differences dependent on which of two commercial albumins was used in the incubations. Differences in albumins used, in rat heart perfusion studies, likely contributed to incongruities between two recent reports describing the fate of phospholipids following VLDL catabolism by cardiac lipases (14, 15). In one study, the greater part of radiolabeled VLDL phosphatidylcholine transferred to the LDL (d 1.019–1.050 g/ml) density range (15), while in the other most was recovered in the HDL (d 1.04–1.21 g/ml) (14). The albumin used in the latter study was described as containing small amounts of phospholipid. Increasing phospholipid content of various commercial albumins also has been described to correlate positively with ability of individual albumins to enhance sterol release from tissue culture cells (33). It seems prudent, therefore, to characterize a given albumin and its effects before embarking on experimentation in which the albumin may exert an independent and unexpected influence.

Mechanisms to explain differences between results obtained with albumin B and C were defined in part by our experiments. The excess of phospholipid in albumin B relative to albumin C seemingly should suffice to account for increased phospholipid recoveries in albumin B incubations in both "LDL" and "HDL". Addition of equivalent amounts of phospholipid however, to incubations using albumin C could not duplicate results obtained with albumin B, suggesting that apoproteins released from the surface of VLDL upon triglycerides hydrolysis (34) were unable to complex with phospholipid under the conditions studied.

Only with addition of exogenous apoproteins (taken from albumin B) and phosphatidylcholine to albumin C could the differences between albumins be markedly diminished. The inability to achieve identical behavior, however, may reflect: *1*) differences between egg yolk lecithin added to albumin C mixtures and phospholipids contained in albumin B; *2*) inability to recover all non-albumin proteins present in al-

bumin B or partial losses during their isolation, so that not all contaminating proteins in albumin B were added to albumin C; *3*) differences in rate of triglyceride lipolysis, hydrolysis of phospholipid, and other unknown factors. Attempts to equalize results by removing either lipid or proteins from albumin B were less successful than addition of these components to albumin C. We believe this reflects inadequacy of our techniques in removing all non-albumin contaminants from albumin B, as shown by the persistence of phospholipid in albumin B after repeated delipidations, and of apoprotein A-I in in vitro-produced lipoproteins produced with albumin B after treatment to remove lipid-binding proteins.

Recent studies in one of our laboratories with purified bovine milk lipoprotein lipase clearly show that some albumin preparations decrease rates of triglyceride hydrolysis in model triolein-gum arabic emulsions, although these differences are less marked when a phospholipid triglyceride emulsion (Intralipid) is used as a substrate for the lipase. Further, these inhibitory effects are much less marked in the presence of cofactor proteins (e.g. apoprotein C-II).[8] The incubation conditions employed in the present study were identical to those reported previously where we showed that solely by the action of lipoprotein lipase on VLDL, we could produce "LDL"-like particles floating in the LDL density range (9). As in our previous study excess lipase was used to ensure maximum triglyceride hydrolysis 30–40 min after addition of the lipase with both albumins. We cannot discount the possibility, however, that differences in initial rates of triglyceride and/or phospholipid hydrolysis between the two albumins play a role in the disparities observed.

The lipoproteins produced in vitro from VLDL show differences related to the albumin used. Both "LDL" and "HDL" were enriched with phospholipid in incubations in which albumin B, itself rich in phospholipid and apoprotein, was used. In our previous report we gave evidence that "LDL" contained an excess of "surface constituents" (9). It was suggested that at least some of this excess surface floated in the "LDL" density range as phospholipid-rich liposomes distinct from the spherical "LDL" particles (9). The albumin-related differences between phospholipid content of the different "LDL" implies that some of these excess "surface components" must derive from the albumin itself. With albumin C which has little, if any, contaminating phospholipid (Table 3) relative phospholipid composition was significantly less. Nevertheless, even when albumin B was used VLDL surface phospholipid contributed to formation of particles in both the "LDL" and "HDL" density range. Using rat VLDL with endogenously radio-^{32}P-labeled phospholipid, triglyceride lipolysis resulted in redistribution of labeled phospholipid into "LDL" and "HDL" (14, 35). Moreover, parallel experiments using labeled rat VLDL in incubation with the two albumin preparations showed that with albumin B about twice the amount of endogenously labeled phospholipid was recovered in the HDL density range.[9] This indicates that the discrepancies between phospholipid recoveries with the different preparations are not solely derived from phospholipids associated with albumin but also phospholipid from the VLDL surface. Thus in VLDL incubations with albumin B, the yield of "HDL" remains more than with albumin C incubations, even after subtracting the two thirds of the phospholipid that could be accounted for by albumin contamination.

The increased relative amounts of free cholesterol in lipoproteins produced from incubations with albumin C reflect decreasing solubilizing phospholipids as VLDL phospholipid undergoes lipolysis. Under normal circumstances, hydrated phospholipids solubilize cholesterol up to a 1:1 ratio (mol/mol) (36). Under the conditions used, about two thirds of VLDL phosphatidylcholine was hydrolyzed to lysolecithin during VLDL lipolysis, leaving less phospholipid to solubilize remaining free cholesterol. Hence, phospholipids in in vitro lipoproteins produced with albumin C are rich with free cholesterol. In the case of albumin B contaminating excess phospholipid is relatively unsaturated with free cholesterol, and solubilizes some of VLDL-free cholesterol, allowing formation of particles with lower cholesterol to phospholipid ratios. Since increasing amounts of free cholesterol inhibit binding of HDL apoproteins to phospholipid (37, 38), this excess in free cholesterol may contribute to the smaller quantities of "HDL" produced with albumin C. It is possible that with albumin C redundant surface remnants that dissociate from VLDL have no sink for free cholesterol, remain relatively saturated with free cholesterol, and are unable to complex with apoproteins.

Despite these differences, incubation of VLDL with lipoprotein lipase in the presence of either albumin, results in formation of lipoprotein-like particles in vitro. The core remnant of VLDL forms a cholesteryl ester–apoprotein B-rich particle in the "LDL" density range, and VLDL surface remnants form phospholipid-rich discoidal particles in the

[8] Bengtsson, G., S. Eisenberg, and T. Olivecrona. To be published.

[9] Eisenberg, S., M. Fainaru, and R. Deckelbaum. Unpublished data.

"HDL" density range. The lipoprotein-poor protein fraction of human plasma ($d > 1.21$ g/ml) in fact contains more lipid than either albumin used in these studies (39). We believe differences in results obtained with these two commercial albumins provide important clues for further studies of VLDL catabolism and subsequent formation of both LDL and HDL.

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